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(54) Title: BACTERIAL POLYPHENOL OXIDASE FROM BACILLUS FOR USE IN OXIDATION OF COLORED SUBSTANCES  (57) Abstract  A novel polyphenol oxidase is produced extracellularly by bacteria, particularly from the genus <i>Bacillus</i> . The enzyme is useful for bleaching and oxidizing a variety of substrates.			

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**BACTERIAL POLYPHENOL OXIDASE FROM BACILLUS FOR USE IN OXIDATION OF COLORED SUBSTANCES****TECHNICAL FIELD**

The present invention relates to a bacterial polyphenol oxidase, to a process of making the enzyme, to a bacterial strain that produces the enzyme, and to processes using the enzyme. More specifically, the present invention provides a novel enzyme source of polyphenol oxidase for use in the oxidation of colored substances and oxidation of polyphenol-containing substances and also for use in cleaning.

**10 BACKGROUND ART**

Polyphenol oxidase and laccase are known as enzymes that oxidize polyphenols. Various studies have been made on the use of polyphenol-oxidizing enzymes. For example, WO 94-29510 reports the delignification in the field of paper and pulp; and WO 15 91-05839, EP 91610032, DE 4008894 and JP-A 64-60693 report the use of the enzymes for bleaching in laundry washing.

However, the only known microbial sources for polyphenol oxidase and laccase are mold fungi such as *Basidiomycetes* and *Deuteromycetes*, and it is generally difficult to cultivate large amounts of such fungi and, in addition, the rate of growth of the cells during cultivation is not high. Improving the enzyme yield by mutation or by use of genetic engineering technology is more difficult with these fungi than with bacteria, since the fungi have complicated life cycles and have complicated gene structures comprising introns, etc. For these reasons, it is difficult to stably and inexpensively obtain large amounts of polyphenol oxidases from such fungi, and bacteria-derived polyphenol oxidases are desired in order to apply them to practical use.

30 It is the object of the present invention to provide a polyphenol oxidase to be produced by bacteria, the bacteria that produce the enzyme and the use of the enzyme, thus providing a novel enzyme source of polyphenol oxidase for use in the

oxidation of colored substances and oxidation of polyphenol-containing substances and also for use in cleaning.

#### STATEMENT OF THE INVENTION

We, the present inventors have assiduously searched 5 various bacteria for extracellular products that catalyze the oxidation of polyphenol substances. Though being extremely difficult, our search for such products has at last resulted in the finding of the fact that bacteria belonging to the genus *Bacillus* can extracellularly produce the intended enzyme. On the 10 basis of this finding, we have completed the present invention.

Accordingly, the present invention provides the following:

A polyphenol oxidase which is derived from a bacterium.

A method for oxidizing a phenolic compound, an alkoxy group-containing aromatic compound, a halogenated phenolic 15 compound or an aromatic amine compound, which comprises treating said compound with said polyphenol oxidase in the presence of oxygen.

A method for bleaching a colored substance, which comprises treating the colored substance with said polyphenol 20 oxidase in the presence of oxygen.

A method for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, which method comprises treating the wash liquor with said polyphenol oxidase in the presence of 25 oxygen.

A method for bleaching colored waste water, which comprises treating the colored waste water with said polyphenol oxidase in the presence of oxygen.

A method for inactivating a microorganism or virus, which 30 comprises treating the microorganism or virus with said polyphenol oxidase in the presence of oxygen.

A method for bleaching of lignin-containing material, which comprises treating the lignin-containing material with said polyphenol oxidase in the presence of oxygen.

35 A detergent composition comprising said polyphenol oxidase.

A method for producing a polyphenol oxidase, which comprises cultivation of a polyphenol oxidase-producing bacterium of the genus *Bacillus* in a suitable nutrient medium, followed by recovery of the polyphenol oxidase.

5 *Bacillus licheniformis* strain SD3003 (FERM P-15383) which is productive of polyphenol oxidase.

Now, the present invention is described in detail hereinunder.

#### DETAILED DESCRIPTION OF THE INVENTION

##### 10 Enzyme-producing Bacteria

According to the invention, polyphenol oxidase is derived from a bacterial strain, preferably a strain of the genus *Bacillus*. Any and every strain of the genus *Bacillus* having the ability to produce polyphenol oxidase can be used herein to obtain the polyphenol oxidase of the invention, and there is not any other specific limitation on the bacteria to be used herein. The enzyme-producing bacteria employable herein include, for example, those of *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus firmus*, *Bacillus licheniformis*, *Bacillus natto*, *Bacillus pumilus*, *Bacillus sphaericus*, *Bacillus subtilis*, etc. Some preferred species are *B. licheniformis*, *B. natto* and *B. sphaericus*, particularly *B. licheniformis*. Some preferred strains are *Bacillus* sp. NCIB 10314, *B. licheniformis* NCIB 8059, NCIB 8061, ATCC 6634, ATCC 25 9945a, ATCC 11945 and SD3003, *B. natto* SN AKU 0205 and *B. sphaericus* IFO 3341.

The strains NCIB 10314, NCIB 8059 and NCIB 8061 are freely available from National Collections of Industrial and Marine Bacteria Limited (NCIMB, previously NCIB), 23 St. Machar Drive, 30 Aberdeen AB2 1RY, Scotland, United Kingdom.

The strains *B. licheniformis* ATCC 6634, ATCC 9945a and ATCC 11945 are freely available from American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, United States of America.

The strain *B. natto* SN AKU 0205 is freely available from the culture collection of the Agricultural Faculty of Kyoto University, Kyoto, Japan.

The strain *B. sphaericus* IFO 3341 is freely available from the Institute for Fermentation (IFO), 17-85 Juso-honmachi 2-chome, Yodogawa-ku, Osaka 532, Japan.

The strain *B. licheniformis* SD3003 was deposited as FERM P-15383 on 28 December 1995 at the National Institute of Bioscience and Human-Technology, Ministry of International Trade and Industry, 1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan. The deposit was subsequently transferred to an international deposit under the Budapest Treaty on 28 January 1997 under the deposit number FERM BP-5801. The deposit was made by Showa Denko K.K., Japan, and is being assigned to Novo Nordisk A/S. The following results were found from morphological observations and physiological tests with this representative strain according to the invention:

Property	Results
Morphology	rod shaped
Gram-staining Ability	+
Spores	+
Shape	Oval
Position	Central to Semi-peripheral
Sporangia	No Evagination
Motility	+
Behavior Toward Oxygen	Anaerobic to Aeration
Catalase	+
Growth in Anaerobic Condition	+
V-P Reaction	+
pH in V-P Broth	5.2
Production of Acid from Glucose	+
Production of Gas from Glucose	-
Liquefaction of Gelatin	+
Decomposition of Starch	+

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Utilization of Citrate	+
Utilization of Propionate	+
Egg Yolk Reaction	-
Reduction of Nitrate	+
Growth at pH 6.8 (in nutrient broth)	+
Growth at pH 5.7	+
Growth in the Presence of 5 NaCl	+
Growth in the Presence of 7 NaCl	+
Growth at 10°C	-
Growth at 30°C	+
Growth at 55°C	+
Growth at 65°C	-
GC content (mol%) of Intracellular DNA(*)	46

(\*) Measured through HPLC.

From the above-mentioned results and with reference to "Bergery's Manual of Systematic Bacteriology", Vol. 2, 1986, 5 Williams & Wilkins, and "The Genus *Bacillus*", 1973, U.S. Department of Agriculture, this strain is classified as *Bacillus licheniformis* SD3003.

#### Preparation of Enzyme

The polyphenol oxidase of the present invention can be obtained by cultivating the cells of strains belonging to the genus *Bacillus* such as those mentioned hereinabove, or those of mutants thereof. In addition, it can also be obtained by cultivating transformants from such strains to be prepared through genetic engineering. For example, host cells as 15 transformed with an expression vector to be prepared by inserting a DNA that codes for the polyphenol oxidase of the invention, along with suitable promoter, operator and terminator DNAs that function to make said enzyme-encoding DNA express the enzyme in host organisms, into a DNA vector having a

replication-initiating point at which the replication of said vector in host organisms is initiated; or host cells as transformed through integration of a DNA that codes for the polyphenol oxidase of the invention with a DNA of a host cell 5 along with suitable promoter, operator and terminator DNAs that function to make said enzyme-encoding DNA express the enzyme in host organisms are cultivated under the condition under which the intended polyphenol oxidase can be expressed, and the thus-expressed polyphenol oxidase is collected from the culture of 10 the thus-cultivated transformant cells.

To obtain a DNA fragment that codes for the polyphenol oxidase of the invention, any ordinary methods are employable. For example, the cDNA or genome library extracted from the 15 strains as referred to hereinabove may be used as the DNA sources. Using, as the probe, an oligonucleotide as synthesized on the basis of the amino acid sequence of the intended enzyme, polyphenol oxidase of the invention, or on the basis of the amino acid sequence of a known polyphenol oxidase, the intended DNA fragment may be specifically identified. Apart from these, 20 clones that express enzymatic activity may be selected, or clones capable of producing a protein that reacts with an antibody to the enzyme, polyphenol oxidase of the invention may be selected.

The polyphenol oxidase of the present invention can be 25 obtained by cultivating the enzyme-producing cells on a conventional synthetic medium or nutrient medium comprising an organic carbon source and an organic nitrogen source. It is desirable to add to the medium a metal salt of  $Cu^{2+}$  ion at a concentration of from 0.001 mM to 10 mM, preferably from 0.01 mM 30 to 1 mM. It is also desirable to add thereto a metal salt of  $Mn^{2+}$  ion at a concentration of from 0.001 mM to 100 mM, preferably from 0.01 mM to 10 mM. The cultivation temperature may be from 20 to 60°C, preferably from 30 to 55°C. The cultivation time may be suitably from 20 hours to 200 hours, 35 preferably from 40 hours to 150 hours.

The polyphenol oxidase as secreted by the cells can be recovered by any ordinary means. The means of recovering the enzyme may comprise a series of steps of separating the cells

from the medium by centrifugation, filtration or membrane separation, followed by purifying the enzyme through chromatography such as ion-exchange chromatography or the like. Concentrating by use of an ultrafiltration membrane is also effective for the recovery of the enzyme. In addition, it is also possible to isolate and concentrate the enzyme through salting-out with ammonium sulfate or the like.

#### Properties of Enzyme

The polyphenol oxidase of the invention preferably has one or more of the following properties: Optimum reaction pH about 7, optimum reaction temperature between 60°C and 80°C, and a molecular weight of about 51,000 (as measured by GFC).

One typical example of the polyphenol oxidase of the present invention is the enzyme derived from *Bacillus licheniformis* SD3003, which catalyzes oxidation within a broad pH range of from 5 to 9, but preferably from 6 to 8, more preferably about pH 7 (see Fig. 1). Thus, this enzyme is characterized in that it catalyzes oxidation within a neutral pH range. The optimum temperature for the action of the enzyme may be from 60°C to 80°C (see Fig. 2). The activity after 30 minutes heat treatment at various predetermined temperatures at pH 7 shows a residual activity of almost 100 at 70°C (Fig. 3). After the enzyme has been treated in a buffer having varying pH values at 30°C for 30 minutes, its activity is still stable in a broad pH range (see Fig. 4). These results ensure the stable activity of the enzyme to catalyze oxidation in various solutions within a broad pH range that covers from weakly acidic to weakly alkaline, at medium to low temperatures. The enzyme was found to have a molecular weight of about 51,000 through GFC analysis.

The polyphenol oxidase of the present invention can be used in combination with any ordinary enzyme that has optimum reaction pH at acidic pH. A combination of a conventional polyphenol oxidase having optimum reaction pH in the acidic range and the polyphenol oxidase of the present invention may make it possible to effect polyphenol oxidase reaction within a broad pH range covering from acidic conditions to weakly alkaline conditions. For the combination of these enzymes for

the intended object, the ratio of the activity amount of the polyphenol oxidase that is most active at acidic pH values to that of the polyphenol oxidase of the present invention to be mixed with the former may be preferably from 1/10 to 10/1 (on 5 activity or enzyme protein basis), more preferably from 1/3 to 3/1. In order to attain the polyphenol oxidase reaction within such a broad pH range, the polyphenol oxidase of the present invention is useful.

#### Method of Measuring Enzyme Activity

10 To determine the polyphenol oxidase in the present invention, the enzyme was reacted with 20 ppm of syringaldazine in an aqueous solution comprising 100 mM Bis-Tris-HCl buffer (pH 7.0) (Bis-Tris is available from Dotite Reagent Co.) at 20°C, and the absorbance at 525 nm of the resulting reaction 15 mixture was measured. The activity amount of the enzyme that oxidizes 1 nmol of syringaldazine per minute is defined herein as 1 munit (hereinafter referred to as mU). The polyphenol oxidase of the invention is generally used at a concentration of from 10 to 500 mU/ml in the treatment of polyphenol-containing 20 substances.

#### Use of Enzyme for Bleaching

The polyphenol oxidase of the invention is useful for oxidizing various substrates, particularly phenolic compounds, and for bleaching various colored substances in the presence of 25 oxygen. Thus, the polyphenol oxidase of the invention finds application for bleaching of dye in solutions.

The polyphenol oxidase of the invention also finds application for dye transfer inhibition, e.g. for treatment of dyed textiles (cf. e.g. WO 92/18687) or during laundering (cf. 30 e.g. WO 91/05839). Thus, the polyphenol oxidase of the invention may be used for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, the method comprising treatment of the wash liquor with the polyphenol oxidase of the invention, 35 optionally in the presence of an additional oxidisable

substrate. The textile dye may be a synthetic dye such as an azo dye, or a natural or nature-identical dye.

The polyphenol oxidase of the present invention is useful in the field of cleaning and bleaching. Thus, the present invention provides the use of the bacteria-derived enzyme in said field. The use of polyphenol oxidases in bleaching is disclosed in, for example, WO 91-05839, DE 4008894 and JP-A 64-60693.

Oxidative bleaching with hydrogen peroxide is widely employed at present in cleaning and washing. However, the bleaching activity of hydrogen oxide is not satisfactory at low temperatures of 60°C or lower. In order to overcome this problem, hydrogen peroxide is used along with peracid precursors. However, the bleaching activity of the combination is still not satisfactory at temperatures of 40°C or lower. Therefore, more effective bleaching systems are desired.

The polyphenol oxidase of the present invention can be combined with one or more of conventional oxidizing agents that have heretofore been used for oxidative bleaching, such as air, oxygen, ozone, hydrogen peroxide, hydrogen peroxide precursors, peracid precursors and peracids, thereby promoting the oxidative bleaching with the enzyme. Thus, the enzyme of the present invention can be effectively combined with any conventional oxidizing agents to enhance its oxidizing and bleaching activity.

The polyphenol oxidase of the present invention can be combined with one or more substances having peroxidase activity, such as peroxidase, lignin peroxidase, manganese peroxidase, etc., thereby promoting the oxidative bleaching with these enzymes.

Of the above-mentioned oxidizing agents, hydrogen peroxide precursors are dissolved in water to generate perhydroxyl ions. These substances include, for example, mono-hydrated or tetra-hydrated perborates, percarbonates, perborax, sodium perpyrophosphate, perbenzoic acid, urea-H<sub>2</sub>O<sub>2</sub> reaction products, melamine-H<sub>2</sub>O<sub>2</sub> reaction products, citric acid perhydrate, etc. Of these, especially preferred are perborates and percarbonates. As the hydrogen peroxide precursor, it is also possible to employ a

hydrogen peroxide-generating system comprising oxidase and a substrate for the enzyme. Examples of the oxidase of this type include glucose oxidase, alcohol oxidase, glycerol oxidase, amine oxidase, amino acid oxidase, D-amino acid oxidase, aryl 5 alcohol oxidase, aldehyde oxidase, galactose oxidase, sorbose oxidase, ureate oxidase, xanthine oxidase, cholesterol oxidase, etc. Of these, especially preferred are glucose oxidase and alcohol oxidase.

The peracid precursors usable in the present invention are 10 reactive acyl group-containing organic compounds, carboxylates, carboxylic acid anhydrides, acetates, etc. These include, for example, TAED (tetraacetyl ethylenediamine), TAMD (tetraacetyl-methylenediamine), TAGU (tetraacetyl glycoluril), DADHT (diacetyl dioxohexahydrotriazine), SNOBS (sodium nonanoyloxybenzenesulfonate), ISONOBS (sodium isononanoyloxybenzenesulfonate), succinic anhydride, benzoic anhydride, phthalic anhydride, PAG (glucose pentaacetate), and xylose tetraacetate. Of these, preferred are TAED and SNOBS.

The peracids usable in the present invention include, for 20 example, DPDDA (diperoxydodecanedioic acid), diperoxyisophthalic acid, magnesium monoperoxyphthalate hexahydrate, and NAPPA (nonyl amidoperoxyadipic acid).

For use of the present polyphenol oxidase as a bleaching composition, the addition of another oxidizable substrate (for 25 the polyphenol oxidase of the invention) at the beginning or during the washing and/or rinsing process may enhance the bleaching effect of the polyphenol oxidase employed.

#### Additional oxidizable substrate

Examples of such oxidizable substrates are organic 30 compounds such as phenolic compounds, e.g. p-hydroxybenzenesulfonate. Other examples of phenolic compounds which may be used for the present purpose are those given in M. Kato and S. Shimizu, Plant Cell Physiol. 26(7), 1985, pp. 1291-1301 (cf. Table 1 in particular) or B.C. Saunders et al., 35 Peroxidase, London, 1964, p. 141 ff.

In WO 94/12621 other types of enhancing agents are disclosed which may be used for the present purpose, e.g.

phenothiazines or phenoazines or derivatives thereof such as 10-methylphenothiazine, 10-phenothiazine-propionic acid, N-hydroxysuccinimide-10-phenothiazine-propionate, 10-ethyl-4-phenothiazine-carboxylic acid, 10-ethylphenothiazine, 10-propyl-5 phenothiazine, 10-isopropylphenothiazine, methyl-10-phenothiazinepropionate, 10-phenylphenothiazine, 10-allylphenothiazine, 10-(3-(4-methyl-1-piperazinyl)propyl)phenothiazine, 10-(2-pyrrolidinoethyl)phenothiazine, promazine, 2-chloro-10-methylphenothiazine, 2-acetyl-10-methylphenothiazine or 10-methylpheno-10 zine.

In WO 96/10079 another group of enhancing agents are disclosed which may be used for the present purpose, e.g., acetosyringone, syringaldehyde, methylsyringate or syringic acid.

15 The amount of oxidizable substrate corresponds to a concentration in the wash liquor of between 0.1  $\mu$ M and 100  $\mu$ M.

#### Detergent composition

The polyphenol oxidase of the present invention can be used in cleaning together with various detergents, cleaners or 20 surfactants. The combination of the enzyme with any of such substances provides cleaner compositions or detergent compositions comprising the polyphenol oxidase of the present invention. Typical examples of cleaners and detergents into which the enzyme of the invention can be combined are cleaner 25 compositions and detergent compositions comprising from 10 to 50% by weight, relative to the weight of the composition, of a surfactant, from 0 to 50% by weight of a builder, from 1 to 50% by weight of an alkaline agent or inorganic electrolyte, from 0.1 to 10% by weight of at least one component selected from the 30 group consisting of re-soiling inhibitors, enzymes, bleaching agents, fluorescent dyes, caking inhibitors and antioxidants.

The surfactants may be any ones that are generally added to cleaners and detergents such as soap, and include, for example, aliphatic sulfates, such as salts of linear or branched 35 alkyl or alkenyl sulfates, salts of amide sulfates, salts of linear or branched alkyl or alkenyl ether sulfates with one or more components of ethylene oxide, propylene oxide and butylene

oxide added thereto; aliphatic sulfonates, such as salts of alkyl sulfonates, salts of amide sulfonates, salts of dialkyl sulfosuccinates, salts of various sulfonates with  $\alpha$ -olefins, vinylidene-type olefins or internal olefins; aromatic sulfonates 5 such as salts of linear or branched alkylbenzenesulfonates; salts or amides of linear or branched alkyl or alkenyl ether carboxylates with one or more components of ethylene oxide, propylene oxide and butylene oxide added thereto; salts or esters of  $\alpha$ -sulfo-fatty acids; amino acid-type surfactants; 10 phosphate-type surfactants, such as acidic alkyl or alkenyl phosphates, salts of alkyl or alkenyl phosphates; sulfonate-type ampholytic surfactants; betaine-type ampholytic surfactants; linear or branched alkyl or alkenyl ethers or alcohols with one or more components of ethylene oxide, propylene oxide and 15 butylene oxide added thereto; polyoxyethylene alkyl or alkenyl phenyl ethers, in which the alkyl or alkenyl moiety may be linear or branched, with one or more components of ethylene oxide, propylene oxide and butylene oxide added thereto; higher fatty acid alkanolamides or alkylene oxide adducts thereof; 20 sucrose fatty acid esters; monoesters of fatty acids with glycerin; alkyl or alkenyl amine oxides; tetraalkylammonium-type cationic surfactants, etc. The counter ion for the anionic surfactants is preferably sodium ion or potassium ion. These surfactants can be used singly or as combined.

25 The builders, alkaline agents and inorganic electrolytes include, for example, phosphates, such as orthophosphates, pyrophosphates, tripolyphosphates, metaphosphates, hexametaphosphates, phytates; salts of phosphonic acids such as ethane-1,1-diphosphonic acid and its derivatives, ethanehydroxy- 30 1,1,2-triphosphonic acid, ethane-1,2-dicarboxy-1,2-diphosphonic acid, methanehydroxyphosphonic acid; salts of phosphono-carboxylic acids such as 2-phosphonobutane-1,2-dicarboxylic acid, 1-phosphonobutane-2,3,4-tricarboxylic acid,  $\alpha$ -methylphosphonosuccinic acid; salts of amino acids such as aspartic acid, 35 glutamic acid; aminopolyacetates, such as nitritotriacetates, ethylenediamine-tetraacetates, diethylenetriamine-pentaacetates; polyelectrolytes, such as polyacrylic acid, polyitaconic acid, polymaleic acid, maleic anhydride copolymers, salts of

carboxymethyl cellulose; non-dissociating polymers, such as polyethylene glycol, polyvinyl alcohol; carboxymethylates of diglycolic acid, oxydisuccinic acid, carboxymethyloxysuccinic acid, gluconic acid, citric acid, lactic acid, tartaric acid, 5 sucrose, lactose or the like; carboxymethylates of pentaerythritol; carboxymethylates of gluconic acid; salts of organic acids such as benzene-polycarboxylic acids, oxalic acid, malic acid, oxydisuccinic acid, gluconic acid; aluminosilicates, such as zeolite; inorganic acids, for example, alkali metal 10 salts of carbonates, sesquicarbonates, sulfates, metasilicates or the like; organic substances, such as starch, urea; inorganic substances, such as sodium chloride, bentonite; and even organic alkaline agents, such as triethanolamine, diethanolamine, monoethanolamine, triisopropanolamine.

15 As has been mentioned hereinabove, the detergent composition of the present invention comprises, as the constitutive components, a surfactant and the polyphenol oxidase of the invention. If desired, it may further contain any of ampholytic surfactants; bleaching agents, such as perborates, 20 percarbonates; dyes; builders; re-soiling inhibitors, such as polyethylene glycol, polyvinyl alcohol, polyvinyl pyrrolidone, carboxymethyl cellulose; caking inhibitors; antioxidants; and various enzymes such as oxidases other than the enzyme of the invention, peroxidases, proteases, lipases, amylases, 25 cellulases.

To incorporate enzymes into the detergent composition of the present invention, any and every method is employable. Preferably, the enzyme is added to the detergent composition in the form of a solution or a non-dusting granulate. Non-dusting 30 granulates can be made, for example, by means of stationary granulation, extrusion granulation, granulation with fluidized beds, centrifugal granulation with fluidized beds and the like. However, the shapes of the enzymes to be added to the detergent composition should not be limited to those as shaped according 35 to such means.

**Treatment of paper and pulp**

Accordingly, the polyphenol oxidase of the invention may be used in a method for bleaching of lignin-containing material, in particular bleaching of pulp for paper production, which 5 method comprises treatment of the lignin or lignin containing material with the polyphenol oxidase enzyme in the presence of oxygen.

For the purpose of delignification and bleaching in pulp production, any of the bacterial strains of the present 10 invention may be inoculated into the material of pulp at any desired step of pulping, thereby producing the polyphenol oxidase of the invention during the step, or a pure product of the enzyme of the present invention may be directly added to wood chips or roughly-beaten pulp. In such manners, the enzyme 15 of the present invention is effectively utilized in the field of bio-pulping and bio-bleaching.

The bleaching of pulp for paper production may be performed in analogy with SE 88/0673 and US 4,690,895. Optionally, the treatment may be carried out in the presence of 20 an additional oxidisable substrate, as described above.

**Waste Water Treatment**

The polyphenol oxidase of the invention also finds application in treatment of waste water e.g. waste water from the chemical or pharmaceutical industry, from dye manufacturing, 25 from dye-works, from the textile industry, or from pulp production (cf. e.g. US 4,623,465, or JP-A-2-31887). Thus, the invention provides a method for treatment of waste water from dye manufacturing, from dye-works, from textile industry, or from pulp manufacturing, the method comprising treatment of the 30 waste water with the polyphenol oxidase in the presence of oxygen.

The polyphenol oxidase of the present invention is useful for the treatment of drainage containing natural substances and artificial substances having polyphenols in their constitutive 35 moieties. The polyphenol oxidase of the invention acts on natural substances of this type, such as flavonoids, xanthones, melamines and other vegetable dyes, as well as lignin. In

addition, the polyphenol oxidase also acts on various toxic AOX substrates, such as dichlorophenol, trichlorophenol, etc.

Optionally, the treatment may be carried out in the presence of an additional oxidisable substrate, as described 5 above.

#### Other Industrial Applications of Enzyme

Further, the polyphenol oxidase of the invention finds application for lignin modification, e.g. in particle board production. Binders for producing wood composites such as fibre 10 boards and particle boards can be made from polyphenol oxidase treated lignin (cf. US 4,432,921). Accordingly, the invention provides a method for enzymatic polymerization and/or modification of lignin or lignin containing material, which method comprises treatment of the lignin or lignin containing 15 material with the polyphenol oxidase in the presence of oxygen.

In addition, polyphenol oxidase of the present invention may be used in biosensors, which reflect the characteristics of the enzyme and are therefore useful for monitoring various aromatic compounds in aqueous solutions or organic solvents at 20 pH falling within a weakly acidic to weakly alkaline range.

Further, since the polyphenol oxidase of the present invention generates active phenoxy radicals, it can be used for efficiently inactivating microorganisms and viruses at pH falling within a weakly acidic to weakly alkaline range. Namely, 25 in addition to the microbicidal activity of the polyphenol oxidase itself of the invention against substrates, the enzyme can exhibit much stronger microbicidal activity while enzymatically generating such active phenoxy radicals. Moreover, even when the substances as microbically processed with the 30 enzyme, polyphenol oxidase of the present invention are then brought into contact with human bodies or are ingested by human beings, or even when they are released in the atmosphere, they are safe, since the substances are, after having been oxidized with the enzyme, converted into safe substances with little 35 toxicity. Thus, the enzyme of the present invention is very useful, as exhibiting its microbicidal activity at any desired

stage to convert the substances processed with it into non-toxic and safe substances.

In addition, the polyphenol oxidase of the present invention is also useful in polymer production where are used 5 the phenoxy radicals and quinones to be derived from the enzyme.

Moreover, where the polyphenol oxidase of the present invention is used to oxidize substances comprising easily-oxidizable polyphenols, such as catechols, it attains both self-oxidation and enzymatic and catalytic oxidation of polyphenols.

10 Thus, the use of the polyphenol oxidase of the present invention for this purpose is extremely effective for attaining efficient oxidation of such substances.

### EXAMPLES

Now, some typical examples of the present invention are 15 mentioned below, which demonstrate the invention more concretely. However, these are to merely exemplify some preferred embodiments of the invention but are not to restrict the scope of the invention.

#### Example 1

##### 20 Cultivation, Crude Purification, Concentration

A 500-ml flask was used herein as the incubator. 100 ml of a medium comprising 0.134  $\text{Na}_2\text{HPO}_4 \cdot 12\text{-H}_2\text{O}$ , 0.03  $\text{KH}_2\text{PO}_4$ , 1 maltose, 1 peptone, 0.1 yeast extract, 0.05  $\text{MgSO}_4 \cdot 7\text{-H}_2\text{O}$ , 0.1 mM  $\text{CuSO}_4$ , 1 mM  $\text{MnCl}_2$  and 2 mM  $\text{CaCl}_2$  was prepared and adjusted to pH 25 7.8 with 20%  $\text{Na}_2\text{CO}_3$  added thereto. Cells of *Bacillus licheniformis* SD3003 (FERM P-15383) were inoculated on the medium, and incubated therein at 50°C for 16 hours while shaking. Then, the temperature of the medium was lowered to 35°C, and the incubation was continued for further 3 days.

30 After the incubation, the culture was centrifuged at 4°C to remove the cells. Ammonium sulfate fractionation was found to be effective for purifying and concentrating the resulting culture broth. More specifically, the culture broth was fractionated with ammonium sulfate at from 20 to 60% of 35 saturation concentration, and most of the active polyphenol

oxidase fraction was collected as the precipitate. The resulting precipitate thus collected through the ammonium sulfate fractionation was dialyzed against 10 mM Bis-Tris-HCl buffer (pH 7.0), and the resulting dialysate was purified and concentrated 5 through ultrafiltration. Thus was obtained an aqueous, crudely-purified concentrate of the enzyme (800 mU/ml) in the fractions falling within a molecular weight range of from 10,000 to 100,000.

**Example 2:**

10 **Substrate Specificity**

The aqueous, crudely-purified concentrate of the enzyme as obtained in the previous Example 1 was tested for the substrate specificity to the oxidation of polyphenol compounds. More specifically, the enzyme concentrate was added to 0.05 mM of 15 each substrate shown in Table 1 below, in 100 mM of Bis-Tris-HCl buffer (pH 7.0) at room temperature (20°C). The difference in the oxygen consumption between the test sample comprising the enzyme concentrate and the control sample not comprising it was determined. The results obtained are shown in the following 20 Table.

Substrate	Oxidation
Syringaldazine	+
4-Anisidine	+
O-phenylenediamine	+
Ferulic Acid	+

**Example 3**

**Molecular Weight**

25 The molecular weight of the enzyme prepared above was determined by GFC (gel filtration chromatography).

The aqueous, crudely-purified concentrate of the enzyme obtained in Example 1 was analyzed and fractionated by HPLC using a series of two GFC columns (Shodex PROTEIN KW-802.5) 30 equilibrated with 1.34 Na<sub>2</sub>PO<sub>4</sub>·12-H<sub>2</sub>O, 0.3 KH<sub>2</sub>PO<sub>4</sub> and 1 NaCl at a flow rate of 1.0 ml/min using a UV detector (at 280 nm), and the

activity of the fractionated enzyme was measured. As a result, the polyphenol oxidase activity peak was eluted within a molecular weight range of from 46,000 to 56,000. MW-Marker (HPLC) produced by Oriental Industrial Co. was used as molecular weight marker protein.

**Example 4:**

**Incubation in 5-Liter Incubator, Concentration, Crude Purification**

Three liters of a medium comprising 0.134  $\text{Na}_2\text{HPO}_4 \cdot 12\text{-H}_2\text{O}$ , 10 0.03  $\text{KH}_2\text{PO}_4$ , 1 maltose, 1 peptone, 0.1 yeast extract, 0.05  $\text{MgSO}_4 \cdot 7\text{-H}_2\text{O}$ , 0.1 mM  $\text{CuSO}_4$ , 1 mM  $\text{MnCl}_2$  and 2 mM  $\text{CaCl}_2$  was prepared and adjusted to pH 7.8 with 10 NaOH. This was put into a 5-liter incubator. Cells of *Bacillus licheniformis* SD3003 (FERM P-15383) were inoculated on the medium, and incubated 15 therein at 50°C for 16 hours while shaking. Then, the temperature of the medium was lowered to 35°C, and the incubation was continued for further 3 days. After the incubation, the culture was centrifuged at 4°C to obtain a cell-free culture broth.

20 Next, a part of this culture broth was fractionated in a mini-tank ultrafiltration system (produced by Millipore Co.) using a mini-tank filter packet (Catalogue No.: PTGCOMP04, produced by Millipore Co.) to obtain a fraction having a molecular weight of 10,000 or higher, which was then 25 concentrated. The resulting concentrate was dialyzed against 200 ppm  $\text{NH}_4\text{HCO}_3$ , and then lyophilized to obtain a crudely-purified, dry lyophilisate. The polyphenol oxidase activity of the dry lyophilisate was 500 mU/mg.

**Example 5:**

**30 Washing of Soiled Fabric with Enzyme-containing Detergent**

0.1 g of the dry lyophilisate as obtained in Example 4 was added to 10 g of a standard detergent comprising 25 by weight of sodium linear alkylbenzenesulfonate (LAS), 5 by weight of polyoxyethylene lauryl ether, 15 by weight of sodium tripolyphosphate, 6 by weight of sodium silicate, 1 by weight of sodium carboxymethyl cellulose and 48 by weight of  $\text{Na}_2\text{SO}_4$ , to

prepare an enzyme-containing detergent sample. The detergent not containing the enzyme was referred to as a control detergent sample.

0.2 ml of 100 ppm Evans' Blue (commercially-available from 5 Wako Pure Chemical Industry Co.) was applied onto the center of white cotton cloths (5 cm x 5 cm) to prepare soiled swatches..

One soiled swatch was put into a 500-ml beaker along with 10 ml of water, and 10 mg of either the enzyme-containing detergent sample or the control detergent sample with no enzyme. 10 Then, the beaker was shaken for 12 minutes to wash the cloth. After thus having been washed, the swatch was rinsed with water and dried in air, and its color was measured with a color-difference meter (CR-200, produced by Minolta Co.) to determine the Y, y and x values of the cloth. From these, the Z value 15 thereof was obtained according to the equation,  $Z = (1 - x - y)Y/y$ .

The results demonstrated an increase of 1.5 points in the degree of whiteness of the swatch treated with the enzyme-containing detergent sample, compared with that treated with the 20 control detergent sample with no enzyme.

**Example 6:**

**Plate test for polyphenol oxidase in various strains**

The following strains were tested: *B. licheniformis* NCIB 8059, NCIB 8061, ATCC 6634, ATCC 9945a, ATCC 11945 and SD3003 25 and *B. natto* SN AKU 0205.

Each strain was cultivated for 3 days with shaking at 34°C in a medium of the following composition at pH 8.6: 0.5 malic acid, 0.5 peptone, 0.05  $MgSO_4 \cdot 7H_2O$ , 0.01 mM  $CuSO_4$ , 0.05 mM  $MnCl_2$ , 0.1 mM  $CaCl_2$ .

30 Each culture broth was tested in a plate assay, using plates with the following composition: 50 mM buffer (neutral pH), 100 ppm  $MgSO_4 \cdot 7H_2O$ , 1.8 agar and 25 ppm syringaldazine as an indicator. The culture broth was applied to a paper disk settled on the plate.

35 For each of the above strains, a distinct pink zone was observed around the paper disk indicating the presence of polyphenol oxidase activity.

**Example 7:****Assay of polyphenol oxidase for various strains**

A well of a 96-well micro-titer dish was filled with 20  $\mu$ l buffer (1M, pH 7.1), 160  $\mu$ l water, 20  $\mu$ l supernatant of culture 5 broth prepared as in the previous example using the strains shown below, and 20  $\mu$ l syringaldazine solution (200 ppm in 50 ethanol). This was incubated for 5 hours at room temperature, and the absorbance at 550 nm was measured before and after the incubation. The results are shown as the increase in absorbance 10 for each strain:

Strain	Increase of absorbance
IFO 3341	.002
NCIB 10314	.004
NCIB 8059	.023
NCIB 8061	.010
ATCC 6634	.024
ATCC 9945a	.052
ATCC 11945	.047

These results confirm a positive polyphenol oxidase activity for the *Bacillus* strains.

**ADVANTAGES OF THE INVENTION**

As has been described in detail hereinabove, the present 15 invention provides a polyphenol oxidase produced by bacteria. It has been found that this enzyme attains enzymatic oxidation and can be used for oxidative treatment of polyphenol substances and colored substances and can be used for the purpose of cleaning and bleaching.

According to the method of the present invention, the polyphenol oxidase of the invention can be produced highly 20 efficiently.

*Bacillus licheniformis* SD3003 of the invention is effectively used for the production of the polyphenol oxidase of 25 the invention.

The present invention also provides a method of using the enzyme, polyphenol oxidase of the invention for processing

colored substances, for processing paper, pulp and fibers, for bleaching and cleaning various substances, and for processing microorganisms and viruses to make them non-toxic.

**BRIEF DESCRIPTION OF THE DRAWINGS**

5 Fig. 1 shows a pH profile for DS 3003-derived polyphenol oxidase.

Fig. 2 shows a temperature profile for DS 3003-derived polyphenol oxidase.

10 Fig. 3 shows a graph showing the temperature-dependent stability of DS 3003-derived polyphenol oxidase.

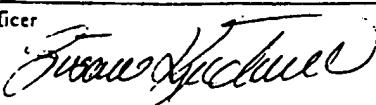
Fig. 4 shows a graph showing the pH-dependent stability of DS 3003-derived polyphenol oxidase.

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22  
INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

<p><b>A. The indications made below relate to the microorganism referred to in the description on page <u>4</u>, lines <u>7-15</u></b></p>	
<p><b>B. IDENTIFICATION OF DEPOSIT</b> <span style="float: right;"><input type="checkbox"/> Further deposits are identified on an additional sheet</span></p>	
<p>Name of depositary institution <b>National Institute of Bioscience and Human-Technology</b></p>	
<p>Address of depositary institution (including postal code and country) <b>1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken 305, Japan</b></p>	
<p>Date of deposit <b>28 January 1997</b></p>	<p>Accession Number <b>FERM BP-5801</b></p>
<p><b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) <span style="float: right;"><input type="checkbox"/> This information is continued on an additional sheet</span></p> <p>Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). After grant of a European patent, in those designated states providing for this "expert option," such option is also hereby requested. And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71.</p>	
<p><b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)</p>	
<p><b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)</p> <p>The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")</p>	
<p>For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer </p>	<p>For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>

## CLAIMS

1. A polyphenol oxidase which is derived from a bacterium.
2. The polyphenol oxidase of claim 1, which is derived from a bacterium of the genus *Bacillus*.
3. The polyphenol oxidase of the preceding claim, wherein the bacterium of the genus *Bacillus* belongs to *B. licheniformis*, *B. natto* or *B. sphaericus*.
4. The polyphenol oxidase of claim 2 or 3, which is obtainable from *Bacillus sp.* NCIB 10314, *B. licheniformis* NCIB 8059, NCIB 8061, ATCC 6634, ATCC 9945a, ATCC 11945 or SD3003 (FERM P-15383), *B. natto* SN AKU 0205 or *B. sphaericus* IFO 3341.
5. The polyphenol oxidase of the preceding claim, which is obtainable from *B. licheniformis* SD3003 (FERM P-15383).
- 15 6. The polyphenol oxidase of any preceding claim, which has the following characteristics:
  - a) Optimum reaction pH of about 7.
  - b) Optimum reaction temperature between 60°C and 80°C.
  - c) Molecular weight of about 51,000 (by gel filtration chromatography).
- 20 7. A method for oxidizing a phenolic compound, an alkoxy group-containing aromatic compound, a halogenated phenolic compound or an aromatic amine compound, which comprises treating said compound with the polyphenol oxidase of any of claims 1 to 6 in the presence of oxygen.

8. A method for bleaching a colored substance, which comprises treating the colored substance with the polyphenol oxidase of any of claims 1 to 6 in the presence of oxygen.

9. A method for inhibiting the transfer of a textile dye from a dyed fabric to another fabric when said fabrics are washed together in a wash liquor, which method comprises treating the wash liquor with the polyphenol oxidase of any of claims 1 to 6 in the presence of oxygen.

10. A method for bleaching colored waste water, which comprises treating the colored waste water with the polyphenol oxidase of any of claims 1 to 6 in the presence of oxygen.

11. A method for inactivating a microorganism or virus, which comprises treating the microorganism or virus with the polyphenol oxidase of any of claims 1 to 6 in the presence of oxygen.

12. A method for bleaching of lignin-containing material, which comprises treating the lignin-containing material with the polyphenol oxidase of any of claims 1 to 6 in the presence of oxygen.

20 13. The method of any of claims 7-12 wherein the polyphenol oxidase is used together with an additional oxidizable substrate.

14. The method of any of claims 7-13 wherein the polyphenol oxidase is used together with a substance having peroxidase activity.

25 15. The method of any of claims 7-13 wherein the polyphenol oxidase is used together with an oxidase and a substrate for the oxidase.

16. A detergent composition comprising the polyphenol oxidase of any of claims 1 to 6.

17. A method for producing a polyphenol oxidase, which comprises cultivation of a polyphenol oxidase-producing bacterium of the genus *Bacillus* in a suitable nutrient medium, followed by recovery of the polyphenol oxidase.

5 18. The method of the preceding claim, wherein the bacterium belongs to *B. licheniformis*, *B. natto* or *B. sphaericus*.

19. The method of the preceding claim, wherein the bacterium is *B. licheniformis* NCIB 8059, NCIB 8061, ATCC 6634, ATCC 9945a, ATCC 11945 and SD3003 (FERM P-15383), *B. natto* SN AKU 0205, *B. 10 sphaericus* IFO 3341 or a mutant of any of these.

20. *Bacillus licheniformis* strain SD3003 (FERM P-15383).

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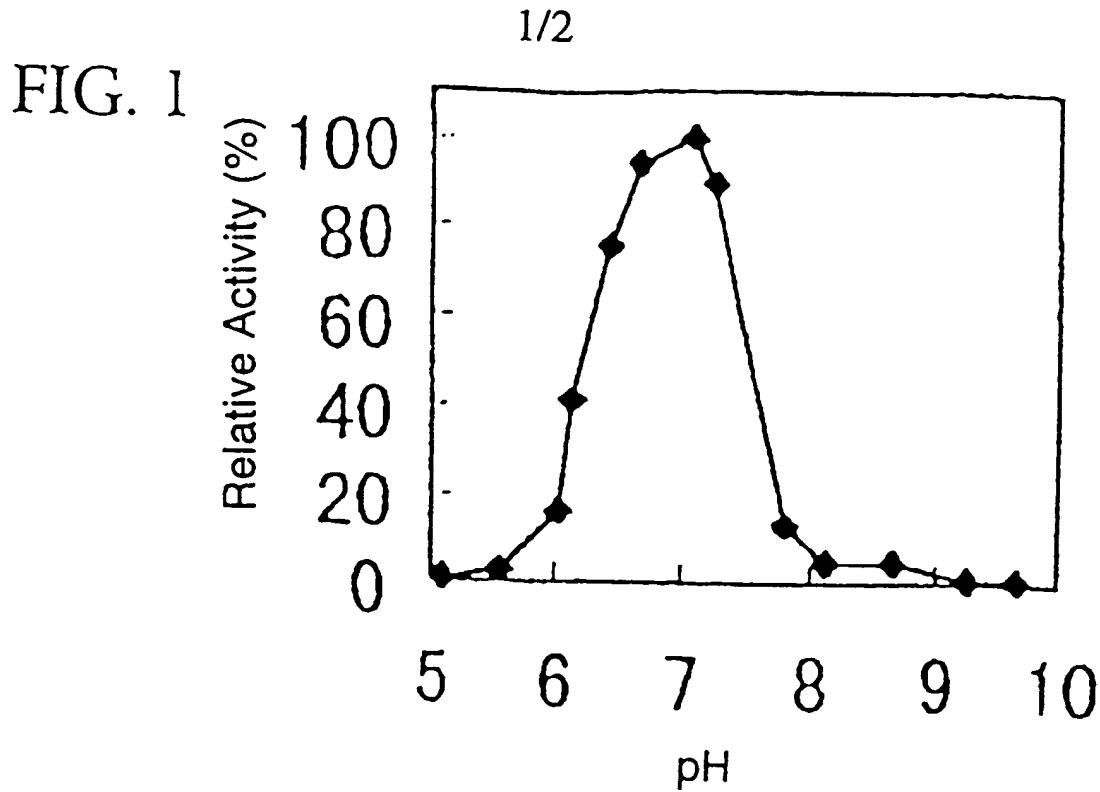
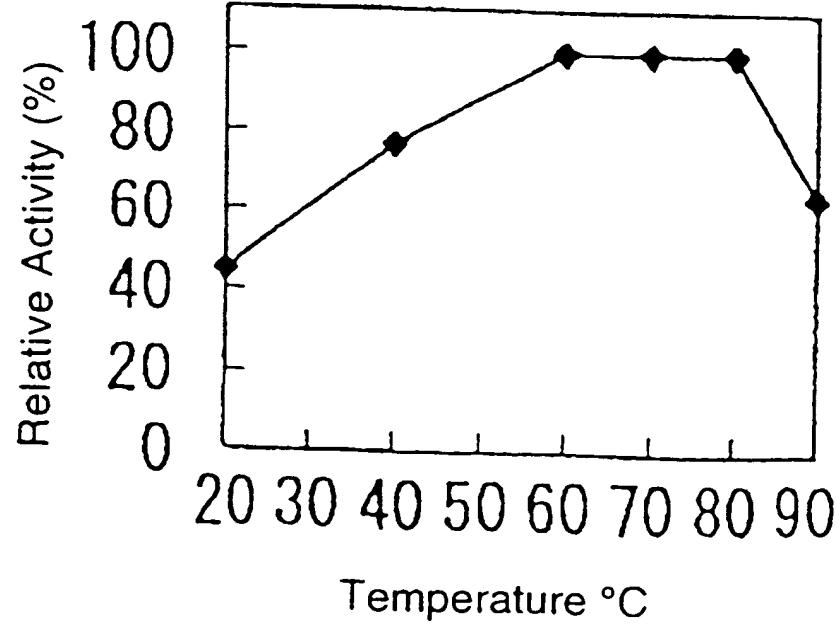


FIG. 2



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FIG. 3

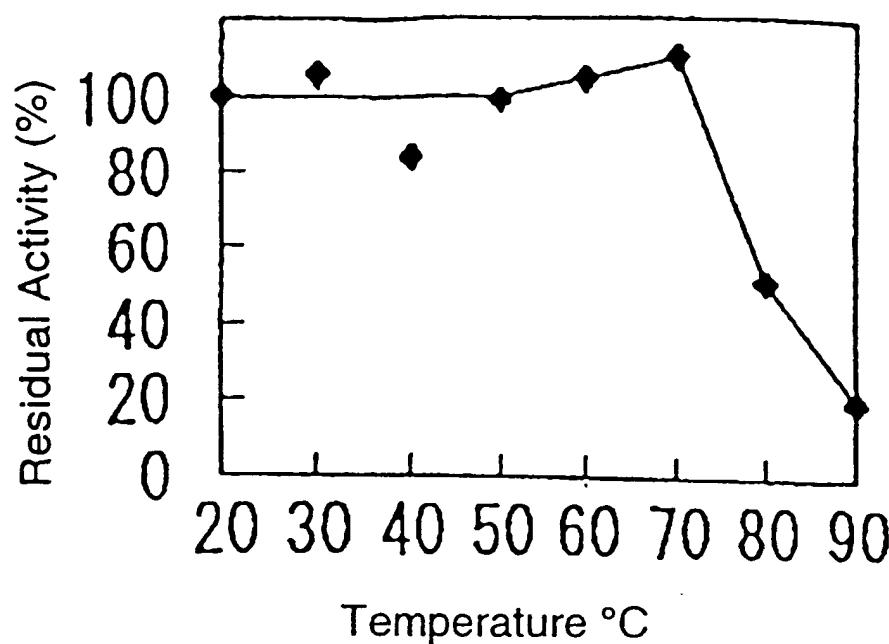
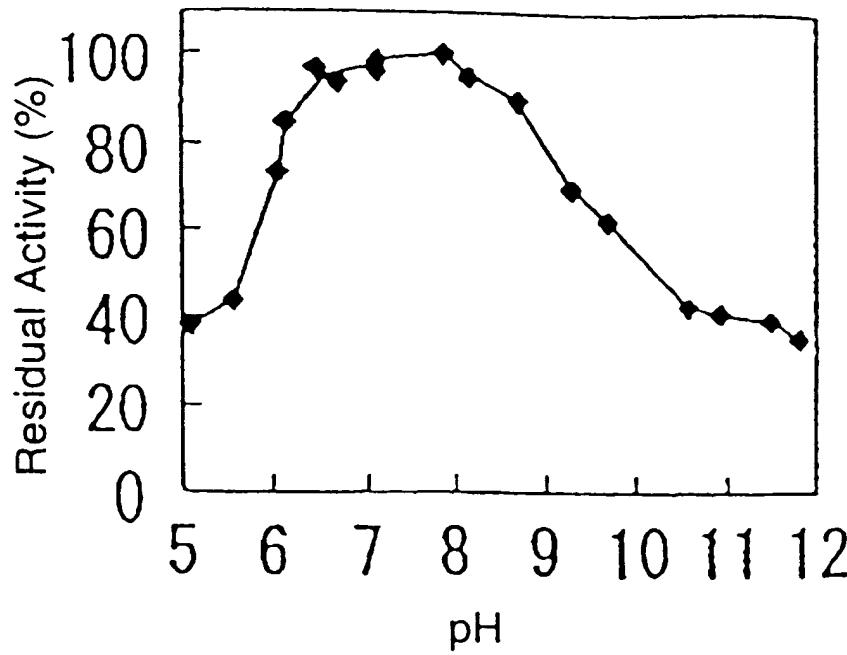


FIG. 4



## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00038

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 9/02, D06L 3/02, C11D 3/386, C02F 3/34, C12N 1/20 // (C12N 9/02, C12S 3/04, C12R 1/07), (C12N 1/20, C12R 1/10)  
According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE, DK, FI, NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 34:SciSearch, Dialog accession no. 13790482, KIM IC et al: "Characterization of the <i>Bacillus-Stearothermophilus</i> BR219 Phenol Hydroxylase Gene"; & Applied and Environmental Microbiology, 1995, V61, N4 (APR), p1252-1256	1,2,7,17
Y	--	8-10,12-16
X	Dialog Information Services, File 34:SciSearch, Dialog accession no. 11729180, DONG FM et al: "Molecular-Cloning and Mapping of Phenol Degradation Genes from <i>Bacillus-Stearothermophilus</i> FDTP-3 and their Expression in <i>Escherichia-Coli</i> "; & Applied and Environmental Microbiology, 1992, V58, NB (AUG), p 2531-2535	1,2,7,17

 Further documents are listed in the continuation of Box C. See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be of particular relevance	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"B" earlier document but published on or after the international filing date	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O" document referring to an oral disclosure, use, exhibition or other means	"&" document member of the same patent family
"P" document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search	Date of mailing of the international search report
13 May 1997	22-05-1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer  Carl-Olof Gustafsson Telephone No. +46 8 782 25 00

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/DK 97/00038

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Information Services, File 5, BIOSIS PREVIEWS Dialog accession no. 5933285, Biosis no. 84065850, Gurujeyalakshmi G et al: "Degradation of Guaiacol Glyceryl ether GGE by <i>Bacillus-Subtilis</i> "; & <i>Appl Microbiol Biotechnol</i> 26 (3). 1987. 289-293  --	1,2,7,17
X	Dialog Information Services, File 5, BIOSIS PREVIEWS Dialog accession no. 6425522, Biosis no. 85026043, Gurujeyalakshmi G et al: "Dissimilation of Ferulic Acid by <i>Bacillus-Subtilis</i> "; & <i>Curr Microbiol</i> 16 (2). 1987. 69-74  --	1,2,7,17
X	National Library of Medicine (NLM), file Medline, Medline accession no. 86082261, Prabhakaran K et al: A possible metabolic role for o-diphenoloxidase in <i>Mycobacterium leprae</i> "; & <i>Experientia</i> 1985 Dec 15;41(12):1571-2  --	1
X	Dialog Information Services, File 5, BIOSIS PREVIEWS Dialog accession no. 11584875, Biosis no. 98184875, Faure D et al: "Comparative study of substrates and inhibitors of <i>Azospirillum lipo-</i> <i>ferum</i> and <i>Pyricularia oryzae</i> laccases"; & <i>Applied and Environmental Microbiology</i> 61 (3). 1995. 1144-1146  --	1
Y	WO9105839 A1 (NOVO NORDISK A/SET AL), 2 May 1991 (02.05.91), page 7, line 1 - line 7  --	1-3,6-9, 12-16
X	Dialog Information Services, File 5, BIOSIS PREVIEWS, Dialog accession no. 7184416, biosis no. 88107161, Perestelo F et al: "Bioalteration of Kraft Pine Lignin by <i>Bacillus-Megaterium</i> isolated from Compost Piles"; & <i>J Ferment Bioeng</i> 68 (2). 1989. 151-153  --	1-3
1-3	  --	,7-17

## INTERNATIONAL SEARCH REPORT

International application No.

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## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5356437 A (GITTE PEDERSEN ET AL), 18 October 1994 (18.10.94), column 3, line 24 - line 37 --	1-3,6-9, 12-16
Y	WO 9323600 A1 (NOVO NORDISK A/S), 25 November 1993 (25.11.93) -- -----	12-15

INTERNATIONAL SEARCH REPORT  
Information on patent family members

International application No.  
PCT/DK 97/00038

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 105839 A1	02/05/91	NONE		
US 5356437 A	18/10/94	DK	9200118 U	23/10/92
		JP	6506731 T	28/07/94
		EP	0580707 A	02/02/94
		WO	9218687 A	29/10/92
WO 9323600 A1	25/11/93	DE	4216276 A	18/11/93